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Detection of Lignin Peroxidase and Xylanase by Immunocytochemical Labeling in Wood Decayed by Basidiomycetes†

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The white rot fungi used in this study caused two different forms of degradation. *Phanerochaete chrysosporium*, strain BKM-F-1767, and *Phellinus pini* caused a preferential removal of lignin from birch wood, whereas *Trametes* (*Coriolus*) *versicolor* caused a nonselective attack of all cell wall components. Use of polyclonal antisera to H8 lignin peroxidase and monoclonal antisera to H2 lignin peroxidase followed by immunogold labeling with protein A-gold or protein G-gold, respectively, showed lignin peroxidase extra- and intracellularly to fungal hyphae and within the delignified cell walls after 12 weeks of laboratory decay. Lignin peroxidase was localized at sites within the cell wall where electron-dense areas of the lignified cell wall layers remained. In wood decayed by *Trametes versicolor*, lignin peroxidase was located primarily along the surface of eroded cell walls. No lignin peroxidase was evident in brown-rotted wood, but slight labeling occurred within hyphal cells. Use of polyclonal antisera to xylanase followed by immunogold labeling showed intense labeling on fungal hyphae and surrounding slime layers and within the woody cell wall, where evidence of degradation was apparent. Colloidal-gold-labeled xylanase was prevalent in wood decayed by all fungi used in this study. Areas of the wood with early stages of cell wall decay had the greatest concentration of gold particles, while little labeling occurred in cells in advanced stages of decay by brown or white rot fungi.

White rot basidiomycetes produce extracellular enzymes that degrade all cell wall components of wood. The sequence and rate in which lignin, cellulose, and hemicellulose are removed from wood varies among different species of fungi. Selective delignification is characteristic of decay by certain strains of *Ganoderma applanatum*, *Phanerochaete chrysosporium*, *Phellinus pini*, *Phlebia tremellosa*, and many others (2, 10, 11, 14, 17, 41). These fungi preferentially degrade large amounts of lignin from wood, with only slight or no loss of cellulose and moderate losses of hemicellulose (8, 42). Other white rot fungi, such as *Trametes versicolor*, some strains of *P. chrysosporium*, *Phlebia subserialis*, and others, are not selective and remove all cell wall components simultaneously (8, 10, 40, 42). Lignin may be removed slightly in advance of cellulose degradation, but extensive loss of cellulose and hemicellulose usually accompanies or immediately follows lignin removal (13). There are also other forms of white rot degradation that result in different patterns of cell wall attack depending on the type of substrate, chemical composition of the wood, and species of white rot fungus involved (11, 30, 41). Brown rot fungi, in contrast, depolymerize cellulose rapidly, and extensive degradation of cellulose and hemicellulose occurs (9, 39). Lignin loss is slight, but a considerable amount of lignin modification takes place (25, 33).

Enzymes involved in degrading lignin have been discovered (35, 50). Two major groups of lignin-degrading enzymes, lignin peroxidases (also referred to as ligninases) and manganese-dependent peroxidases, have been characterized (22, 29, 35, 45). Multiple isozyme forms of lignin peroxidase exist. All are glycoproteins and appear to have similar catalytic activity and molecular masses (29, 31). Two isozymes, H8 and H2, dominate in the extracellular culture fluid of *P. chrysosporium*. Lignin peroxidase is also produced by other white rot fungi, and current investigation suggests that lignin peroxidase is a key enzyme of white rot fungi produced during the ligninolytic growth phase (32). Other enzymes are also undoubtedly involved in the process of lignin degradation (22, 32, 45). Mn-dependent peroxidase is a heme protein that acts as a classical peroxidase (45). Multiple molecular forms of this enzyme also have been found that oxidize Mn(II) to Mn(III), resulting in oxidation of various substrates (37). The role of Mn(II)-dependent peroxidases in lignin degradation, however, is not completely understood.

The use of polyclonal and monoclonal antibodies and immunocytochemistry has allowed the detection and localization of fungal metabolites within substrates (43). Fluorescence immunohistochemical detection in wood of two white rot fungi that cause severe root rot, *Armillaria mellea* and *Heterobasidion annosum*, has been successful with polyclonal antisera to homogenized mycelia (52). Detection of a brown rot fungus, *Postia placenta*, with polyclonal antibodies to extracellular fractions and cell wall material of the fungus has also been shown (23). Polyclonal antisera of this type, however, were not species specific, and cross-reactions to other fungi occurred.

The first investigations with immunogold cytochemical investigations by transmission electron microscopy and polyclonal lignin peroxidase antisera have shown that the enzyme is not released extracellularly by *P. chrysosporium* (21, 38). Lignin peroxidase was only found intracellularly in the cytoplasmic area close to and bound with the fungal cell plasmalemma. These results were difficult to interpret since

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ligninase could be obtained from extracellular culture fluids of white rot fungi (33), and therefore ligninase also should be found outside of the hyphal cell wall. In addition, if this enzyme were important in lignin degradation, it should be found within cell walls of wood attacked by white rot fungi.

Many factors may affect the cytochemical localization of specific enzymes resulting in negative labeling with gold complexes (7, 48). One important aspect is the effect of tissue processing (i.e., washing, fixation, type of resin, and conditions of embedding), which can introduce changes in tissue components (7). Culture conditions for the fungus within the substrate must be conducive to lignin peroxidase production. In addition, antisera have to be specific for the particular antigen of interest. Recently, Daniel et al. (16) showed extracellular localization of lignin peroxidase in slime layers around fungal hyphae and along areas of cell wall erosion in decayed wood by using polyclonal antisera and immunogold labeling techniques. The strain of *P. chrysosporium* used in the study caused a nonselective attack on cell wall components. This type of white rot resulted in an erosion of the cell wall from the lumen toward the middle lamella. Lignin peroxidase was found localized along the edge of the eroded cell wall but not within the wall. To determine how far enzymes of *P. chrysosporium* would penetrate the cell wall, Strebotnik et al. (48) infiltrated decayed pine wood with a concentrated culture filtrate followed by immunogold labeling for lignin peroxidase. Lignin peroxidase was found on the surface of the cell wall or within areas that were degraded extensively. Enzymes from the culture fluid did not diffuse into nondecayed areas of the cell wall.

Another important aspect of lignocellulose degradation is the role of hemicellulases during lignin degradation. Lignin is intimately associated with hemicellulose in the woody cell wall (28, 44), and hemicellulose loss usually accompanies extensive lignin removal (8, 14, 42). Improved techniques for labeling hemicellulose in wood have been developed (9, 27, 47, 51) and applied to studies of wood decay by white rot fungi (9, 46). These studies demonstrated that hemicellulose is degraded when lignin is removed from wood. Immunogold labeling studies to detect xylanase in wood during decay by fungi have not been completed.

This paper reports the ultrastructural localization of lignin peroxidase H8 and H2 and xylanase by using colloidal-gold immunocytochemistry in birch wood decayed by three white rot fungi, (*Trametes versicolor*, *P. chrysosporium*, and *Phellinus pini*) and one brown rot fungus (*Fomitopsis pinicola*).

MATERIALS AND METHODS

Wood blocks of sound, freshly cut birch, *Betula papyrifera*, were decayed in the laboratory for 12 weeks by the procedures of Otjen and Blanchette (40). Two white rot fungi that preferentially degrade lignin in deciduous wood, *Phanerochaete chrysosporium* BKM-F-1767 and *Phellinus pini* RAB-83-19, one white rot fungus that simultaneously degrades all cell wall components, *Trametes* (*Coriolus*) *versicolor* MAD 697-R, and one brown rot fungus, *Fomitopsis pinicola* RAB-32 were used to inoculate the wood blocks. Noninoculated wood blocks served as controls. The blocks were incubated in the dark at 27°C and 90% relative humidity. With these techniques, a combination of intermediate and advanced decay could be expected within the wood blocks. The chemical and morphological changes that occur in birch wood after decay by these methods have been characterized previously by several different methods (9, 10, 13, 14).

The sound and decayed wood blocks were cut into small segments (approximately 1 by 1 by 0.4 mm) and fixed in 1% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 2 h. Samples were rinsed three times for 20 min each and dehydrated through a graded acetone series. Embedding procedures with Quetol 651 resin (34) were as described previously (1). Samples in resin were cured at 74°C for 8 h and cut with a diamond knife. Sections (100 to 120 nm) were collected on nickel grids and used for immunocytochemical studies. Additional samples were cut from the wood blocks and fixed with 2% $KMnO_4$ in distilled water, followed by dehydration in Quetol 651 and embedment (1).

Polyclonal antiserum against a highly purified xylanase from *Aureobasidium pullulans* Y-2311-1 (T. D. Leathers, J. Indus. Microbiol., in press) was obtained from New Zealand White rabbits. Xylanase has shown amino acid sequence homology to xylanases from taxonomically distant sources (36). Polyclonal anti-lignin peroxidase H8 was also raised in White rabbits and partially purified by ammonium sulfate precipitation (31). Monoclonal antibodies to lignin peroxidase H2 from three distinct cell lines were also used.

For the indirect gold labeling used in this study, a colloidal gold solution, with a gold particle size of 13 to 15 nm, was prepared by the methods of Frens (20). The collodial gold was coupled to protein A (5, 19) for use with sections treated with polyclonal antisera or to protein G (6) for use with the monoclonal antisera.

Samples fixed in glutaraldehyde were used for the immunolabeling studies. Sections on nickel grids were washed in 0.01 M phosphate-buffered saline (PBS) for 10 min at room temperature, followed by incubation in one of the primary antibodies for 1.5 h. Sections were washed three times in 0.01 M PBS, incubated for 45 min in the protein A-gold or protein G-gold solution, and rinsed in distilled water. Antisera were diluted in various concentrations to determine optimum labeling with 100 mM PBS containing 1.4% NaCl, 0.1% Tween 80, and 1% fish gelatin. Controls used for immunolabeling in all procedures included (i) antibodies absorbed for 48 h at 4°C with their respective antigen (H8 or H2 lignin peroxidase, xylanase) in PBS, (ii) preimmune sera used in the protocol instead of the primary antibody, (iii) the primary antibody omitted from the procedure, and (iv) sound wood that was not inoculated with fungi. Grids were examined in a Hitachi 600 transmission electron microscope without poststaining.

RESULTS

Localization of ligninase activity in cell walls of birch wood was observed with polyclonal antisera to ligninase H8 and monoclonal antisera to ligninase H2 followed by immunogold labeling and transmission electron microscopy. The white-rot fungi used in this study caused two different forms of degradation. *T. versicolor* caused a nonselective attack on the wood cell walls (Fig. 1a), whereas *P. pini* and *P. chrysosporium* caused a selective removal of lignin (Fig. 1b). Major differences were also observed in distribution of gold labeling among the different white rot fungi. Fiber and vessel cell walls from wood decayed by *P. pini* had presumed evidence of lignin peroxidase activity, demonstrated by sites of gold labeling within the cell walls when polyclonal lignin peroxidase H8 antisera were used followed by indirect gold labeling (Fig. 2a and b). At this stage of degradation, lignin within the secondary walls, as well as middle lamella between some cells, had been removed. Intense labeling occurred in cell corners and middle lamella between cells

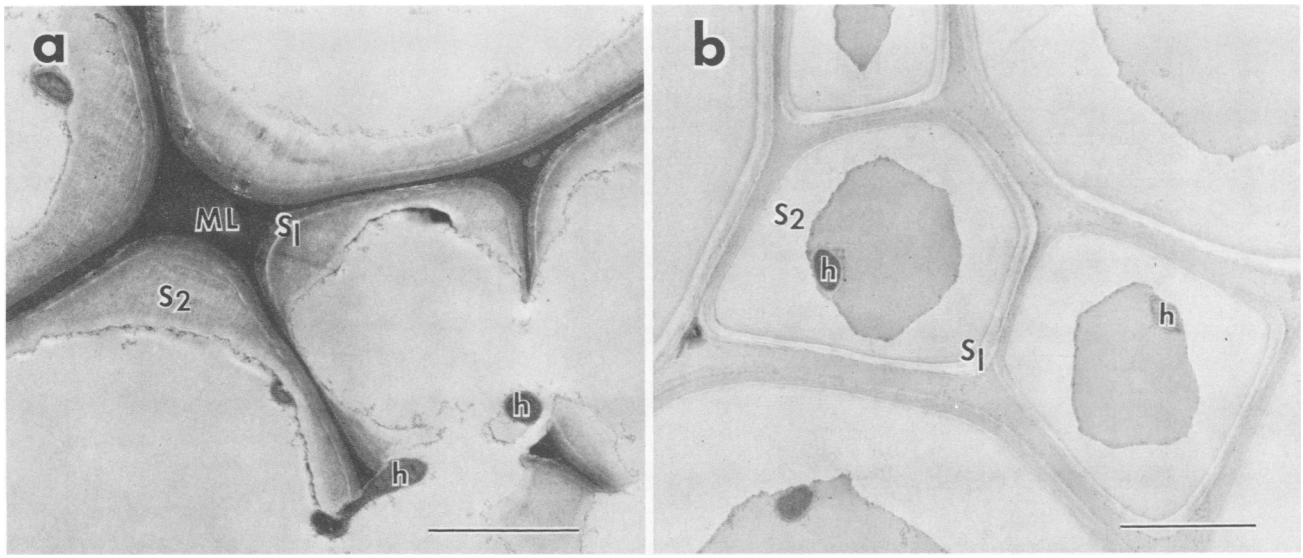


FIG. 1. Transverse sections of birch wood decayed by white rot fungi. $KMnO_4$ fixation was used to stain lignin, appearing as electron-dense areas, in the cells. (a) Simultaneous degradation of all cell wall components by *T. versicolor*. Cell walls are eroded from the lumen toward the middle lamella. (b) Preferential lignin degradation by *P. pini*, showing cells with delignified secondary walls and completely degraded middle lamellae. ML, middle lamella; S_1 and S_2, secondary wall layers; h, hypha. Bars, 4 μm.

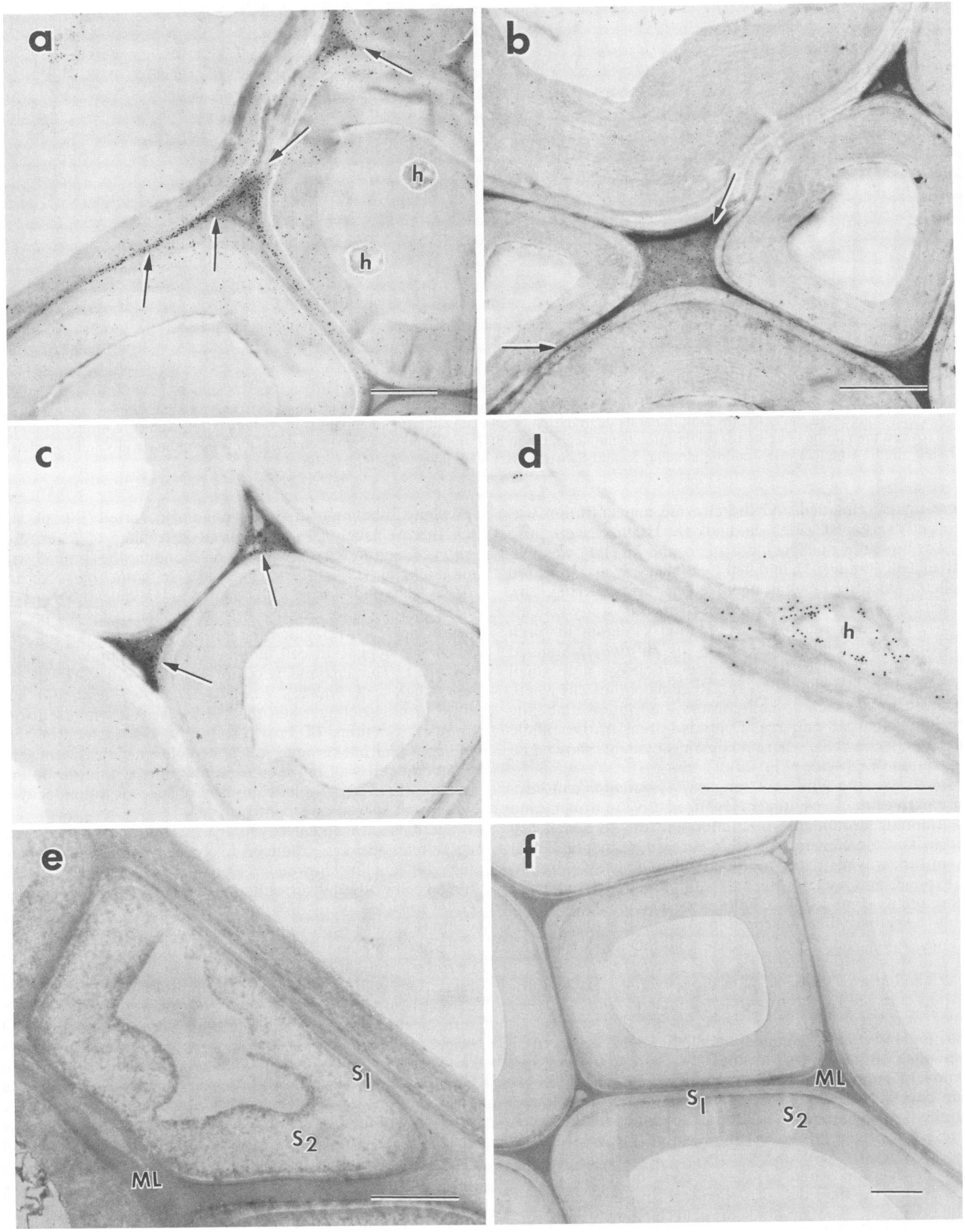
where lignin remained and the enzyme concentration was apparently greatest (Fig. 2a and b). Hyphae were also labeled, but little nonspecific background labeling was evident in cell lumina. Delignified secondary walls appeared swollen, and little labeling occurred in this area (Fig. 2b). The strain of *P. chrysosporium* used in this study, BKM-F-1767, caused a selective degradation of lignin in birch wood that was similar to the decay by *P. pini*. Delignified secondary walls had little electron density and small amounts of gold labeling (Fig. 2c). Regions of the cells where lignin remained, such as cell corners, had intense labeling with polyclonal H8 antisera. Degraded areas of the middle lamella between cells, where no lignin is evident, showed no localization of enzyme (Fig. 2c).

In contrast, the white rot fungus *T. versicolor* caused a nonselective attack resulting in localized erosion troughs and wall thinning around the circumference of the secondary wall surface. Localization of lignin peroxidase by polyclonal H8 antisera was restricted to hyphae, the slime layer around the hyphae, and areas of the cell wall immediately adjacent to the fungus (Fig. 2d). The amount of labeling was considerably less than that observed for wood decayed by *P. chrysosporium* and *P. pini*. Intense gold labeling within the cell wall was not observed.

No labeling was observed with the polyclonal H8 antisera in wood decayed by the brown rot fungus *F. pinicola* (Fig. 1e) or noninoculated birch wood blocks that served as controls. However, some labeling, albeit slight, occurred in the hyphae of the brown rot fungus.

Antisera from three different monoclonal cell lines to lignin peroxidase H2 showed results similar to those found with the polyclonal antisera with immunogold labeling techniques. Enzyme was concentrated within the cell wall of wood decayed by *P. pini* at sites where the middle lamella had been degraded (Fig. 3a). Large concentrations of gold were evident at the edge of delignified and nondelignified middle lamella. The amount of gold labeling, however, varied within degraded walls and among different cells (Fig. 3b). In some cells, only small amounts of gold labeling were present. Labeling of fungal hyphae also varied (Fig. 3b and c). Intense labeling was observed in hyphae of *P. chrysosporium* and in the slime layer surrounding the hyphae with monoclonal H2 antisera (Fig. 3c). In some parts of the decayed wood where cells had less advanced stages of degradation, gold labeling was not found deep within the secondary wall. Enzymes apparently penetrated the cell wall from the hyphae in cell lumina toward the middle lamella (arrows). The enzyme was not found in all parts of the secondary wall (Fig. 3c). Wood decayed by *T. versicolor* had little to no labeling when the H2 antiserum was used (not shown). Sections of brown-rotted wood decayed by *F. pinicola* had background levels of gold particles within the degraded cell wall but increased amounts within the fungal hyphae (Fig. 3d). No labeling was observed in nondecayed wood that served as a control.

Sections of wood decayed by *P. pini* and treated with polyclonal antisera produced to xylanase followed by indirect gold labeling showed large quantities of gold particles on fungal cell walls and surrounding slime layers (Fig. 4a and b). Gold particles were distributed throughout the secondary walls but were more numerous in areas of the cell where delignification had not progressed to an advanced stage (arrows in Fig. 4a). Cell corners were not labeled (Fig. 4b). Increased gold labeling in cell lumina was observed in comparison to sections treated with lignin peroxidase antisera. Sections of decayed wood that exhibited an advanced stage of delignification by *P. pini* or *P. chrysosporium* showed no labeling of remaining secondary walls (Fig. 4c). Gold labeling was evident, however, around the hyphae in these sections. Wood decayed by *T. versicolor* had xylanase activity within the secondary walls, as indicated by the localization of gold particles (Fig. 4d), but labeling was not prevalent within the compound middle lamella. Hyphae of *F. pinicola* also had intense labeling around the circumference of the fungal cell wall, with considerably fewer gold particles within the hyphae (Fig. 4e and f). Wood decayed by the brown rot fungus had some labeling within the inner regions of secondary walls, but no labeling was found in the swollen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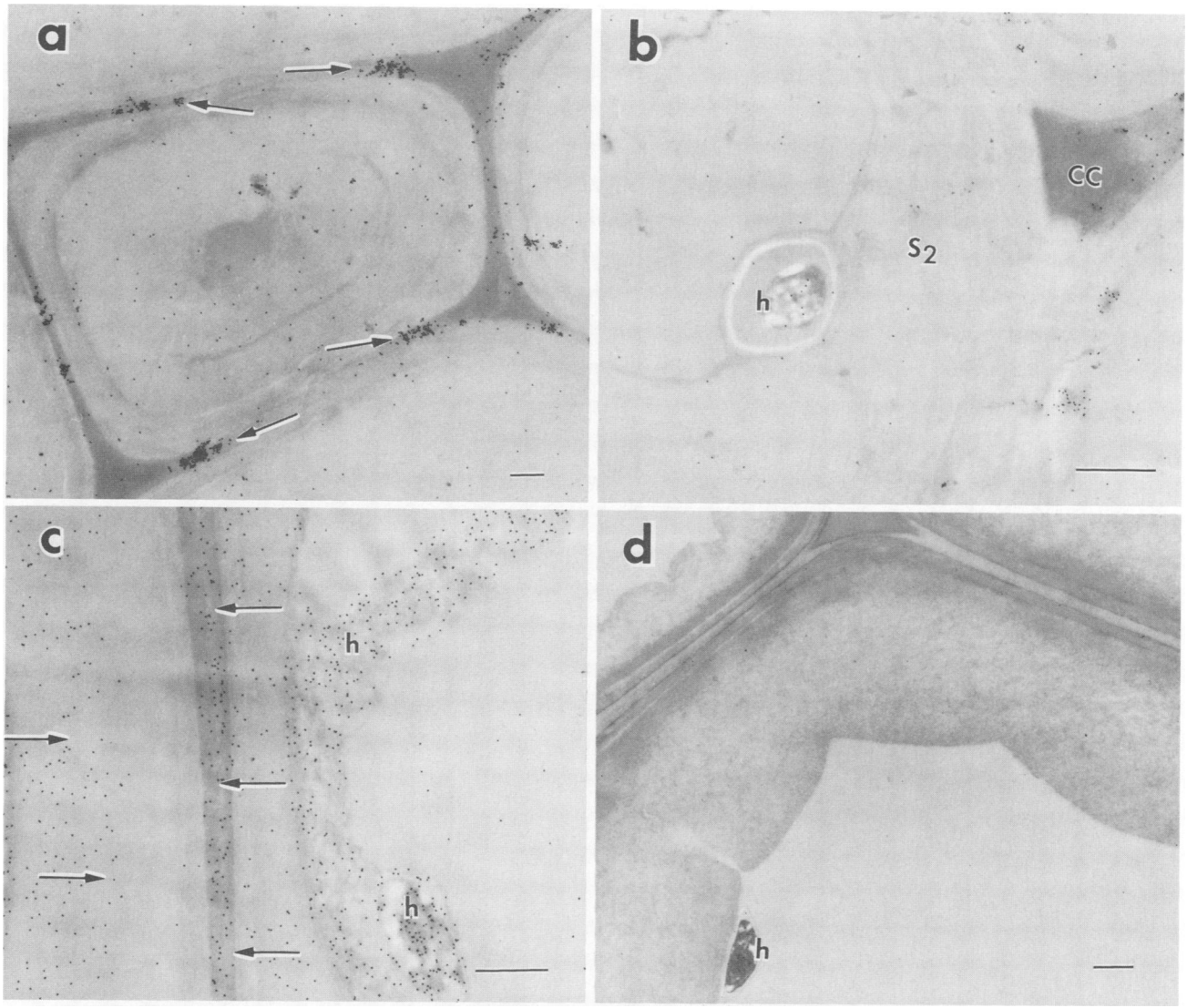


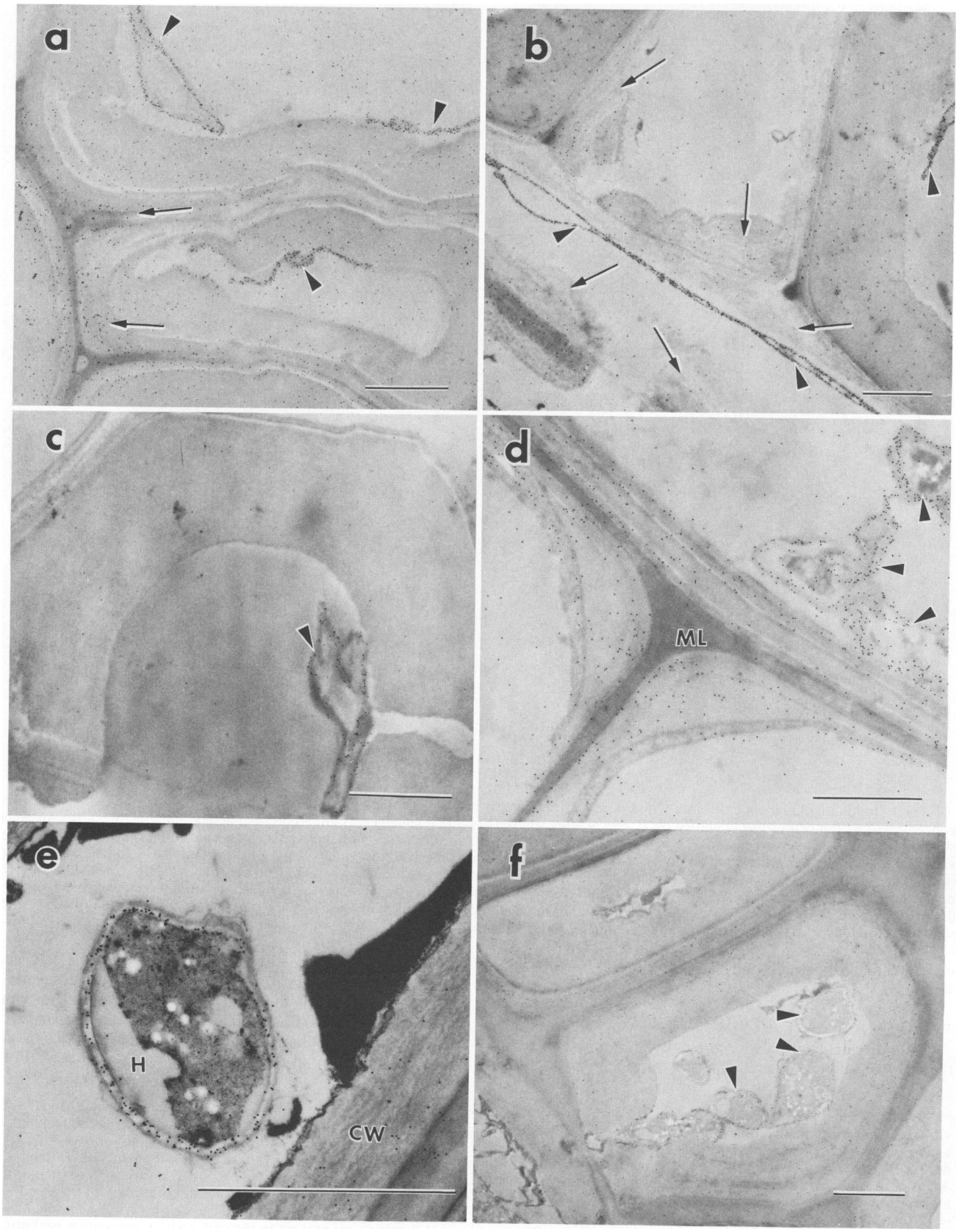
FIG. 3. Immunogold labeling of birch wood decayed by white or brown rot fungi with monoclonal antisera to H2. (a) Gold particles are concentrated within cell walls delignified by *P. pini* at sites where the middle lamella was apparently being degraded (arrows). (b) Advanced stages of *P. pini*-decayed wood, with low concentrations of gold labeling in delignified cell walls. Concentrations of gold particles were variable from cell to cell, depending on the stage of degradation. (c) Wood decayed by *P. chrysosporium*, with intense gold labeling in hyphae (h). Gold particles are evident in the secondary wall and middle lamella of the cell where hypha are located, but penetration of lignin peroxidase into the adjacent cell wall, presumably from the hypha in the adjacent cell lumen, was not deep within the secondary wall (arrows). (d) Brown-rotted wood did not have gold particles within the degraded cell wall, but some labeling was found in the hypha. h, Hypha; S_2, secondary wall layer; cc, cell corner of middle lamella. Bars, 1 μm.

middle lamella between cells and within cell corners (Fig. 4f).

DISCUSSION

Ligninase localization was shown by immunogold labeling techniques to be extracellular and intracellular to fungal hyphae and within cell walls of birch wood that had been decayed by *P. chrysosporium* and *P. pini*. The strains of these two white rot fungi used in this study did not cause continuous degradation of all cell wall components from the lumen to middle lamella, as observed for *T. versicolor*. Instead, preferential degradation of lignin occurred. Previ-

FIG. 2. Immunogold labeling of sound and decayed birch wood with polyclonal antisera to lignin peroxidase H8. (a and b) Cells with various stages of delignification by *P. pini*, showing sites of lignin peroxidase activity within the walls. Secondary walls are delignified and appear swollen. Electron-dense regions of the middle lamella, where lignin remains, are sites of intense gold labeling (arrows). (c) Wood decayed by *P. chrysosporium* BKM-F-1767 shows little gold labeling in secondary wall layers or where the middle lamella was degraded. Areas of the middle lamella that appear lignified (arrows) have a large concentration of gold particles. (d) Wood decayed by *T. versicolor* with moderate gold labeling around the hypha (h) and at sites of eroded cell walls. Large concentrations of gold are not present within the cell wall. (e) Wood decayed by the brown rot fungus *F. pinicola* exhibit extremely degraded cell walls. All secondary wall layers and middle lamella have a swollen appearance and are electron dense. Only a few random gold particles are present in the cell walls, representing background levels. (f) Sound wood with no gold labeling after treatment. ML, Middle lamella; S_1 and S_2, secondary wall layers; h, hypha. Bars, 3 μm.


a
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ous investigations have demonstrated that lignin is removed from the cell wall by *P. pini* and other white rot fungi that delignify wood, first from the secondary wall layers and subsequently from the middle lamellae between cells and then the cell corner regions (11, 13, 41). After 12 weeks of decay in the laboratory, the birch wood used in our study showed areas of extensive delignification. Sites of lignin peroxidase concentration were within the cell wall near electron-dense areas of the middle lamella. These areas corresponded to zones where some lignin remained and which are presumed to be sites of enzyme activity. Noninoculated wood, various controls, and brown-rotted wood were all without gold particles, demonstrating the specificity of the methods and antisera used.

Immunogold-labeling investigations on decayed wood published during the past few years have not shown extracellular lignin peroxidase activity (21, 49). However, in recent publications (16, 48), extracellular lignin peroxidase was found within heavily attacked cell walls and along the surface of eroded cell walls. The isolate of *P. chrysosporium* used by Daniel et al. (16), P 127-1, appeared to cause only simultaneous degradation of cell walls in birch wood, whereas strain BKM-F-1767 used in our study resulted in preferential lignin loss. In the study by Strebotnik et al. (48), the BKM-F-1767 strain was used on pine wood as a substrate, and no evidence of delignification was apparent. In addition, the intensity of lignin peroxidase labeling was weak and results were variable. Recently, different forms of white rot, showing simultaneous removal of all cell wall components and selective lignin removal, have been obtained by using different isolates of *P. chrysosporium* as well as different types of wood substrates (10, 42). Growth and environmental conditions may also affect lignin peroxidase production and activity (4, 31), and the different methods used to decay wood could influence the results.

The localization of lignin peroxidase in wood decayed by *P. chrysosporium* and *P. pini* varied in intensity depending on the stage of cell wall decomposition. After 12 weeks of decay, intense labeling occurred at regions that were most electron dense. These electron-dense areas have been shown in previous reports to be sites where lignin has not been degraded (11, 13, 16). Secondary wall layers that were delignified appeared swollen, and gold labeling was minimal. Both the H8 polyclonal and H2 monoclonal antisera showed similar results for lignin peroxidase with these fungi. However, labeling of H8 was reduced in wood decayed by *T. versicolor*, and no specific labeling was observed with H2 monoclonal antisera compared with the results of other white rot fungi. This may be due to the reduced amount of lignin peroxidase produced by *T. versicolor* after 12 weeks of degradation, or else the specificity of the antisera was not appropriate for enzyme recognition. Although no gold labeling occurred in cell walls of brown-rotted wood, some gold particles were found within fungal hyphae. Greater affinity of the antisera for cell organelles or compounds within the hyphae of *F. pinicola* may have occurred, so that the absorbed colloidal gold-protein G adhered to the hyphae after washing.

Little is known about the role of hemicellulases in wood decomposition (9, 18). The results presented here demonstrate the presence of xylanase within the decayed secondary walls of all samples. Gold particles were concentrated around fungal hyphae and were located in decayed zones of the cell wall. In regions where decay was not advanced, such as the middle lamella (Fig. 4d), no gold particles were found. These results confirm earlier data, determined by using other techniques, that hemicellulose is removed from birch wood when lignin is degraded (3, 9, 14). In cells that had evidence of advanced delignification, gold labeling and xylanase activity were no longer evident. Mapping of xylanase activity during the progressive stages of decomposition should be possible by the methods presented with wood decayed for various time periods. Modification of the protocol, however, may be necessary to decrease the amount of background labeling. The random appearance of gold particles in cell lumina may be due to movement of the enzyme within cells during fixation, dehydration, or embedding procedures. The specificity of the antisera does not appear to be in question, since control sections incubated with preimmune serum were negative and cells with advanced stages of white or brown rot degradation, which would be expected to have little xylanase present, showed no background labeling.

Brown rot fungi depolymerize cellulose and degrade all polysaccharides early in the decay process (15, 33, 39). Brown-rotted wood used in this study had extensive cell wall degradation after 12 weeks of degradation. Wood sugar analyses of birch wood blocks decayed for the same time period indicate that over 70% of the xylose, representing the xylan content of wood, was removed (9). Localization of xylanase at this advanced stage of decay was limited to hyphae and areas deep within the secondary wall (Fig. 4e). The low concentration of gold particles suggests that most xylanase activity had ceased. Highley (25) postulates that hemicellulose is degraded prior to cellulose. Observations during early stages of decay by brown rot fungi, with the immunogold methods presented, should provide a mechanism to visualize the pattern of hemicellulose degradation.

The exact role of lignin peroxidase during the degradation process of lignin remains unclear. Apparently, many other enzymes may also be involved (22, 32, 45). The results presented here demonstrate the close spatial association of lignin peroxidase to areas where lignin is being degraded within the cell wall. The cross-reactivity of antisera from lignin peroxidase of *P. chrysosporium* with other white rot fungi shows that this enzyme or related enzymes are produced by many different fungi when they degrade wood.

FIG. 4. Immunogold labeling of birch wood decayed by white or brown rot fungi with polyclonal antisera to xylanase. (a) Hyphae, especially hyphal surfaces of *P. pini*, were well labeled, and gold particles can be seen outlining the hyphal structure (arrowheads). Although random distribution of some gold particles was evident, greater amounts were present in cells with evidence of less extensive stages of degradation (arrows). (b) Hypha of *P. pini* in delignified cells, showing labeling of hyphae (arrowheads) as well as areas of the cell wall. Fewer gold particles are present in areas of the cell wall with advanced degradation (arrows). (c) In areas of the wood decayed by *P. chrysosporium* that had cells with extensive delignification, few gold particles can be seen within the secondary walls that remained. However, hyphal surfaces were well labelled (arrowhead). (d) Hypha of *T. versicolor*, showing gold labeling in and around the fungal cell (arrowhead), and decayed cell walls with gold particles distributed within the secondary wall but not the middle lamella (ML). (e) Hypha (H) of the brown rot fungus *F. pinicola* in cell lumen, with gold labeling around the circumference of the hypha and within the fungus. Labeling is also evident within the woody cell wall (CW). (f) Cells with advanced stages of brown rot, showing some labeling of areas within the secondary wall but few to no gold particles in the degraded and apparently expanded middle lamella region. Gold particles are still evident on the hyphae (arrowheads). Bars, 3 μm.

Other investigators have also detected lignin peroxidase in other white rot fungi (24, 26, 32).

The localization of lignin peroxidase within the cell wall indicates that this enzyme can diffuse into decayed wood. In white-rotted wood attacked by fungi that cause a nonselective attack, lignin peroxidase was restricted to the superficial regions of the cell walls (16). When high levels of culture filtrate or lignin peroxidase were infiltrated into degraded wood, followed by immunogold labeling, the enzyme was found to penetrate the heavily degraded areas (16, 48). The different morphological and chemical characteristics of wood decayed by fungi that preferentially degrade lignin (13, 14) appear to be responsible for access of lignin peroxidase to areas within the cell walls. Lignin and hemicellulose are degraded extensively, progressing from the cell lumen toward the middle lamella. As this degradation takes place, (i.e., lignin and hemicellulose are removed and remaining cell wall becomes swollen), the physical nature of the cell wall is changed, and avenues for lignin peroxidase are apparently provided so that the enzyme moves into the interior portions of the woody cell wall. Colloidal-gold cytochemistry, coupling purified lignin peroxidase with colloidal gold, has not been successful in labeling native lignin (21). Since lignin peroxidase does not identify native lignin in wood as a substrate, some modification of cell wall components appears necessary. Additional information is needed to determine what modifications are necessary in wood before lignin peroxidase can attack its substrate. The methods presented here, as well as those of others published recently (16, 48), provide the techniques necessary to monitor enzyme localization during the decay process. As new enzymes become available and antisera are prepared, an accurate representation of the major enzymes involved during the process of lignin degradation should become evident.

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